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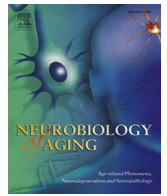
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Genetic variability in *SQSTM1* and risk of early-onset Alzheimer dementia: a European early-onset dementia consortium study



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ABSTRACT

Meta-analysis of existing genome-wide association studies on Alzheimer's disease (AD) showed subgenome-wide association of an intronic variant in the sequestosome 1 (*SQSTM1*) gene with AD. We performed targeted resequencing of *SQSTM1* in Flanders-Belgian AD patients selected to be enriched for a genetic background ($n = 435$) and geographically matched nonaffected individuals ($n = 872$) to investigate the role of both common and rare *SQSTM1* variants. Results were extended to the European early-onset dementia cohorts (926 early-onset Alzheimer's disease [EOAD] patients and 1476 nonaffected individuals). Of the 61 detected exonic variants in *SQSTM1*, the majority were rare ($n = 57$). Rare variant (minor allele frequency <0.01) burden analysis did not reveal an increased frequency of rare variants in EOAD patients in any of the separate study populations nor when meta-analyzing all cohorts. Common variants p.D292= and p.R312= showed nominal association with AD (odds ratio_{p.D292=} = 1.11 [95% confidence interval = 1–1.22], $p = 0.04$), only when including the Flanders-Belgian cohort in the meta-analysis. We cannot exclude a role of *SQSTM1* genetic variability in late-onset AD, but our data indicate that *SQSTM1* does not play a major role in the etiology of EOAD.

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1. Introduction

Mega meta-analysis of existing genome-wide association studies (GWAS) on Alzheimer's disease (AD) performed by the International Genomics of Alzheimer's Project identified an intronic variant in the sequestosome 1 (*SQSTM1*) gene, which showed subgenome-wide association with AD (rs72807343, odds ratio [OR] = 1.35 [95% confidence interval {CI} = 1.20–1.52], $p = 7 \times 10^{-7}$) (Lambert et al., 2013). *SQSTM1* encodes the p62 protein that is a stress-responsive ubiquitin-binding protein commonly found in neuronal cytoplasmic inclusions in protein aggregation diseases like AD, Parkinson disease, Pick disease, etc. (Kuusisto et al., 2001; Zatloukal et al., 2002). P62 is involved in protein degradation via the proteasome, in protein aggregation, and in autophagy (Bjorkoy et al., 2006; Seibenhener et al., 2004). Mutations in this gene, especially affecting the ubiquitin-associated (UBA) domain of the p62 protein, have been found to be the most common cause of Paget disease of the bone (PDB), a disease that is characterized by malformed bones (Johnson-Pais et al., 2003). Using a hypothesis-driven candidate gene approach, a direct genetic role for *SQSTM1* in both familial and sporadic amyotrophic lateral sclerosis was identified in a European-American population (Fecto et al., 2011). Screening of additional amyotrophic lateral sclerosis (ALS) populations led to the identification of novel variations in the gene (Hirano et al., 2013; Teyssou et al., 2013). These results suggested that presumably ALS and PDB share a common molecular pathomechanism (Hirano et al., 2013), reminiscent of PDB, and frontotemporal lobar degeneration (FTLD) in VCP mutation carriers (Kimonis et al., 2008; Watts et al., 2007; van der Zee et al., 2009). Adding to the firmly established clinicopathologic relationship between ALS and FTLD, studies were conducted to investigate the frequency of *SQSTM1* variants in FTLD patients (Rubino et al., 2012; van der Zee et al., 2014). Rare mutations clustering in the UBA domain of p62 were found to be associated with a 2-fold increased risk to develop FTLD (Rubino et al., 2012; van der Zee et al., 2014).

In this study, we investigated the contribution of both rare and common variations in the *SQSTM1* exonic region to the occurrence of AD in a cohort of Flanders-Belgian AD patients selected to be enriched for a genetic background (early disease onset and/or familial AD, $n = 435$) and geographically matched nonaffected individuals ($n = 872$). Our results were extended to a European early-onset dementia (EU EOD) cohort comprising 926 early-onset Alzheimer's disease (EOAD) patients and 1476 nonaffected individuals.

2. Materials and methods

2.1. Study population

2.1.1. Flanders-Belgian cohort

We selected 435 AD patients with early-onset age (age of onset [AAO] <65 years) and/or familial disease (at least one first-degree relative with the disease) (mean AAO = 67.7 ± 8.2 years, women = 62.2%) from a large prospective cohort of Belgian AD patients ascertained at the Memory Clinic of the ZNA Middelheim and Hoge Beuken, Antwerp, Belgium (PPDD and SE) (Engelborghs et al., 2003, 2006) and the Memory Clinic of the University Hospitals of Leuven, Leuven, Belgium (MV and RV) (Table 1). Consensus diagnosis of possible and probable AD was given by at least 2 neurologists based on the National Institute of Neurological and Communication Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association criteria (McKhann et al., 1984, 2011). Each patient underwent a neuropsychological examination and structural and/or functional neuroimaging (Bettens et al., 2009).

The Flanders-Belgian control cohort ($n = 872$, mean age at inclusion = 66 ± 12.7 years, women = 55.6%) consisted primarily of community-dwelling volunteers, for whom subjective memory complaints, neurologic or psychiatric antecedents, and a familial history of neurodegeneration were ruled out by means of an interview. Cognitive screening was performed using the Mini-Mental State Examination (cutoff score ≥ 26) (Folstein et al., 1975). The control cohort additionally included spouses of patients, examined at the Memory Clinic of ZNA Middelheim and Hoge Beuken, Antwerp, Belgium and the Memory Clinic at the University Hospitals of Leuven, Gasthuisberg, Leuven, Belgium.

All participants and/or their legal guardian gave written informed consent for participation in clinical and genetic studies. Clinical study protocol and the informed consent forms for patient ascertainment were approved by the ethics committee of the respective hospitals at the cohort sampling sites in Belgium. The genetic study protocols and informed consent forms were approved by the Ethics Committees of the University of Antwerp and the University Hospital of Antwerp, Belgium.

2.1.2. EU EOD cohort

Patients and control individuals ascertained through the EU EOD consortium were included as replication cohort (van der Zee et al., 2013, 2014). For this study, DNA and medical/demographic

Table 1All exonic variants in *SQSTM1* that were identified in the Flanders-Belgian cohort

Exonic variants	Protein domain	Genomic position	Protein position	dbSNP137	Minor allele count AD	Freq AD	OR (95% CI), <i>p</i> value	Minor allele count control	Freq control	Freq EVS
Exon 1	PB1	g.179248023C>G	p.P29=		0	0.000		2	0.001	—
	PB1	g.179248034C>T	p.A33V	rs200396166	1	0.001		0	0.000	0.0008
	PB1	g.179248119C>T	p.G61=		1	0.001		2	0.001	—
Exon 3	PB1<>ZZ	g.179250885G>A	p.R110H		0	0.000		1	0.001	—
	PB1<>ZZ	g.179250906C>T	p.A117V	rs147810437	0	0.000		2	0.001	0.0012
	PB1<>ZZ	g.179250930A>G	p.N125S		0	0.000		1	0.001	—
	ZZ	g.179251013G>A	p.V153I	rs145056421	1	0.001		3	0.002	0.001
Exon 4	ZZ<>TRAF6	g.179251313G>A	p.T221=		0	0.000		1	0.001	—
Exon 5	TRAF6	g.179252168G>A	p.P232=	rs145688323	1	0.001		0	0.000	0
	TRAF6	g.179252184A>G	p.K238E	rs11548633	8	0.009		7	0.004	0.0035
Exon 6	PEST1	g.179260072A>C	p.R265S		0	0.000		1	0.001	—
	PEST1	g.179260073A>C	p.S266R		0	0.000		1	0.001	—
	PEST1	g.179260099G>C	p.E274D	rs55793208	21	0.025	1.67 (0.91–3.05), 0.096	31	0.018	0.0253
	PEST1	g.179260110C>T	p.T278I	rs200445838	0	0.000		1	0.001	—
	PEST1	g.179260153C>T	p.D292=	rs4935	489	0.575	1.22 (1.01–1.47), 0.035	933	0.535	0.5294
	PEST1<>LIR	g.179260165G>A	p.P296=	rs148984239	0	0.000		1	0.001	0.0001
	PEST1<>LIR	g.179260183C>T	p.G302=	rs11548642	1	0.001		1	0.001	0.0002
	PEST1<>LIR	g.179260202G>A	p.A308=		0	0.000		1	0.001	—
	PEST1<>LIR	g.179260213G>A	p.R312=	rs4797	479	0.564	1.23 (1.02–1.48), 0.03	920	0.528	0.5227
	PEST1<>LIR	g.179260231C>T	p.S318=	rs56092424	20	0.024	1.56 (0.85–2.89), 0.151	31	0.018	0.0212
	PEST1<>LIR	g.179260232G>A	p.E319K	rs61748794	0	0.000		1	0.001	0.0002
Exon 7	LIR	g.179260601G>A	p.S328=	rs146164139	5	0.006		11	0.006	0.0045
	LIR<>PEST2	g.179260649A>G	p.K344=	—	0	0.000		3	0.002	—
	PEST2	g.179260661G>A	p.P348=	rs10058037	0	0.000		1	0.001	0.0002
	PEST2	g.179260700C>T	p.S361=	rs201591177	1	0.001		0	0.000	0.0001
Exon 8	UBA	g.179263445C>T	p.P392L	rs104893941	2	0.002		4	0.002	0.0021
	UBA	g.179263543G>A	p.G425R		0	0.000		1	0.001	—
	UBA	g.179263544G>A	p.A426=		0	0.000		2	0.001	—
	UBA	g.179263583C>T	p.P438L		2	0.002		0	0.000	—
	UBA	g.179263586C>T	p.P439L		0	0.000		1	0.001	—

SQSTM1 (NM_003900.4). Genomic position in base pairs according to hg19 (GRCh37), and “—” denotes not applicable. Total allele count for the Belgian population is 870 AD and 1744 control alleles. A comparison of frequencies with those in EVS in European samples (at least 7388 alleles) is provided. The variations with MAF >1% and significant *p* values are indicated in bold. Chi² *p* values are corrected for AAO, gender, and APOE genotype. Protein domains are based on UniProt information; transcript level on NM_003900.4, and protein level on the GenPept Accession Number NP_003891.1. Protein domains were assigned as described previously (van der Zee et al., 2014). “<>” denotes between protein domains. Rare nonsynonymous variants p.P438L and p.A33V were present in the same AD patient (female, AAO = 75 years). In addition, variants p.R265S and p.S266R were identified in the same control individual (male, AAO = 70 years).

Key: AAO, age at onset; AD, Alzheimer's disease; EVS, Exome Variant Server; freq, frequency; MAF, multiple allele frequency; OR, odds ratio; *SQSTM1*, sequestosome 1; TRAF6, tumor necrosis factor receptor–associated factor 6; UBA, ubiquitin associated.

information on 926 EOAD patients (disease onset = <65 years), originating from Spain (*n* = 329), Portugal (*n* = 107), Italy (*n* = 210), Sweden (*n* = 175), Germany (*n* = 98), and Czech Republic (*n* = 7), was contributed by members of the consortium (Supplementary Table 1). Patients were diagnosed following the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association International Working Group criteria (McKhann et al., 1984, 2011). Diagnosis of pathology confirmed patients was based on currently accepted diagnostic criteria (Montine et al., 2012). Genetic profiling of AD-associated genes was previously generated for a subset of patients *APP* (*n* = 227), *presenilin-1* (*PSEN1*) (*n* = 248), *PSEN2* (*n* = 225), *GRN* (*n* = 11), and *MAPT* (*n* = 11) (Supplementary Materials and Methods). This revealed 3 *APP*, 14 *PSEN1*, and 5 *PSEN2* missense mutations and 1 *GRN* frameshift mutation. Genotyping of *APOE* was performed in the total patient population.

As control group, we sequenced 1476 age- and origin-matched European individuals (Spain [*n* = 484], Portugal [*n* = 127], Italy [*n* = 518], Sweden [*n* = 340], and Czech Republic [*n* = 7]) tested for normal cognition for age and education and Mini-Mental State Examination cutoff score >26. For all EU EOD participants, informed consent for participation, approved by the ethics committee of the respective hospitals or sampling sites, was obtained. A more detailed description of the EU EOD consortium cohort can be found in the Supplementary Table 1.

2.2. *SQSTM1* sequencing

For the Flanders-Belgian cohort, genomic DNA was extracted from peripheral blood lymphocytes using MagDEA DNA Whole-Blood (8Lx) kit (Precision System Science, Pleasanton, CA, USA). Resequencing of the full *SQSTM1* exonic DNA sequence (coding sequence [CDS]) of the Flanders-Belgian sample (*n* = 1307) was performed by polymerase chain reaction (PCR)–based amplification of DNA followed by Sanger sequencing of the 8 exons and intron-exon boundaries (NM_003900.4). Primers were designed using the PCR primer design tool Primer3 (primer sequences are available on request, <http://primer3.sourceforge.net/>). All sequences were analyzed with Seqman (DNASTAR, Madison, WI, USA) and NovoSNP software packages (Reumers et al., 2011; Weckx et al., 2005).

For the EU EOD cohort, DNA samples were subjected to quality control procedures as previously described (van der Zee et al., 2014). Resequencing of *SQSTM1* was performed by massive parallel resequencing after multiplex amplicon enrichment. To this end, we designed a target enrichment assay based on MASTR technology (Multiplicom, Niel, Belgium) covering *SQSTM1* coding exons 2–8, flanking intron-exon boundaries and untranslated regions. *SQSTM1* exon 1 was screened by Sanger sequencing as described previously. Primers for multiplex PCR were designed using mPCR (Multiplicom). Multiplex PCR was performed for amplification of the target region, followed by purification of the equimolar pooled

amplicon libraries using Agencourt AMPureXP beads (Beckman Coulter, CA, USA). Patient-specific barcodes (Illumina Nextera XT) were incorporated in a universal PCR step. Barcoded samples were pooled before bridge amplification and sequencing on an Illumina MiSeq platform, using the Illumina reagent kit v2, generating 250-bp paired-end reads. A subset of the control cohort ($n = 707$) was screened using both MASTR massive parallel resequencing and Sanger sequencing. This dual analysis showed a high concordance of 99.4% between both used technologies.

Fastq-mcf was used to trim the MiSeq (Illumina) adapters of the paired-end reads. Alignment and mapping of the reads against the whole genome (hg19) was performed with Burrows-Wheeler Aligner (Li and Durbin, 2009). Variant calling and annotation was performed using GATK, version 2.2 (McKenna et al., 2010) in combination with GenomeComb software (Reumers et al., 2011). Raw reads of rare variants were manually checked using the integrative genomics viewer (IGV; Broad Institute, Cambridge, MA, USA). Rare variants were validated on genomic DNA using Sanger sequencing. Numbering of variations at genomic DNA level was based on the GenBank Accession Number NC_000005.9, transcript level on NM_003900.4, and protein level on the GenPept Accession Number NP_003891.1.

2.3. In silico prediction

The effects of coding *SQSTM1* variations were predicted using Polymorphism Phenotyping (PolyPhen-2, version 2, <http://genetics.bwh.harvard.edu/pph2/>), Sorting Intolerant from Tolerant (SIFT, http://sift.jcvi.org/www/SIFT_enst_submit.html), and SNPs&Go (<http://snps.uib.es/snps-and-go/snps-and-go.html>). PolyPhen-2 predicts a possible impact of amino acid substitutions on the structure and function of human proteins. The Polyphen-2 score ranges from 0 to 1 and indicates the probability of a damaging effect. SIFT predicts whether an amino acid substitution affects protein function based on sequence homology and physical proportions of amino acids. A SIFT score <0.05 suggests pathogenicity. SNPs&Go predicts human disease-related mutations in functionally annotated proteins. The reliability index reports the reliability of the prediction, scoring from 0 (unreliable) to 10 (reliable). If the disease probability is >0.5 , the variation is predicted as disease associated. MutationTaster was used to predict the effect of synonymous variants (Schwarz et al., 2014). If the probability value is close to 1, this indicates a high certainty of the prediction.

2.4. Statistical analyses

For common *SQSTM1* variants with minor allele frequency (MAF) $>1\%$, deviations from Hardy-Weinberg equilibrium were assessed using an exact Hardy-Weinberg equilibrium test (www.pharmgat.org/IIPGA2/Bioinformatics/exacthweform), and allele frequencies were compared between AD patients and healthy control individuals using χ^2 statistics. ORs (calculated relative to the common genotype) and 95% CIs were calculated using a logistic regression model, using SPSS, version 20.0, for Windows (IBM SPSS Inc, Chicago, IL, USA), corrected for onset age (AAO), gender, and APOE $\epsilon 4$. A 2-sided p -value of 0.05 was considered statistically significant. Fixed-effects (Mantel-Haenszel) meta-analysis of the common variants was performed based on raw allele data of the different EU EOD cohorts. The Czech (7 patients and 7 control individuals) and German (patients only) cohorts were not included in the association analysis. Mantel-Haenszel summary OR and Woolf test for heterogeneity were computed in R using the library rmeta, version 2.16. We performed rare variant burden analysis on the cumulative frequency of nonsynonymous variant alleles with MAF $<1\%$ either spanning the full exonic region of *SQSTM1* or affecting

different protein domains using χ^2 statistics. As for the common variants, meta-analysis (Mantel-Haenszel) of rare variant alleles was performed, following the same procedures as described earlier. Protein domains were assigned as described previously (van der Zee et al., 2014).

3. Results

3.1. SQSTM1 mutation screening in the Flanders-Belgian cohort

Sequencing of the *SQSTM1* CDS in the Flanders-Belgian cohort resulted in the identification of 26 rare variants (MAF <0.01), of which 14 variations were nonsynonymous (Table 1). Two of these variants (p.A33V and p.P438L) were absent from 872 Belgian control individuals. The amino acid substitution p.P438L, located in the C-terminal region of the UBA domain of the protein and predicted to be damaging for protein structure and/or function, was previously described in a patient with ALS (Rubino et al., 2012). The mutation was found in 2 AD patients with onset ages of 67 and 75 years. The 2 AD patients shared a second nonsynonymous variation, p.E274D, which is a low-frequency variant (MAF 0.025). The AD patient with AAO of 75 years also carried a third rare nonsynonymous variant, which is the other variant that was absent from control individuals, that is, p.A33V. This variant is located in the first exon of *SQSTM1*, encoding the Phox and Bem1p (PB1) domain. This variation was absent from our Flanders-Belgian control cohort, but has been reported before at low frequency in public databases, and is predicted benign based on impact on protein structure and function (Supplementary Table 3). Review of clinical records of both patients did not show evidence of ALS or PDB, although on X-ray of the skull of the patient with AAO of 75 years, a diploic skull was noted. Further, 2 synonymous variants (p.P232= and p.S361=) were found in patients only located in tumor necrosis factor receptor-associated factor 6 (TRAF6) and proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST2) domains. Sixteen variants were observed in control individuals only, of which 9 were nonsynonymous.

3.2. Rare variant association analysis in the Flanders-Belgian cohort

No significant difference in total number of rare variations (MAF <0.01) was identified between the Belgian AD (14/870 = 0.016) and control individuals (24/1744 = 0.014) (relative risk [RR] = 0.94 [95% CI = 0.57–1.55], allelic $p = 0.8$) (Table 2). When investigating rare variant burden in the different functional protein domains of *SQSTM1*, we did not observe a significant increase in rare variants in specific domains in AD patients versus control individuals. The low-frequency variant p.E274D (and p.S318=, in strong LD) was observed slightly more often in patients (MAF 0.025) than control individuals (MAF 0.018), but this did not reach statistical significance (OR = 1.67 [95% CI = 0.91–3.05], allelic $p = 0.096$). Inclusion of this variant in the whole gene burden analysis (RR = 1.16 [95% CI = 0.91–1.49], allelic $p = 0.23$) or analysis of the PEST1 domain (RR = 1.2 [95% CI = 0.72–2.16], allelic $p = 0.44$) in which it is located did not change the observations.

3.3. Association of common SQSTM1 variants in the Flanders-Belgian cohort

Two common polymorphisms with MAF >0.05 were observed in the CDS of *SQSTM1*, both synonymous (p.D292= and p.R312=). Allelic association with AD was observed for both variants p.D292= (OR = 1.22 [95% CI = 1.01–1.46], allelic $p = 0.037$) and p.R312= (OR = 1.23 [95% CI = 1.02–1.48], allelic $p = 0.03$) that are in strong pairwise linkage disequilibrium (HapMap $D' = 0.915$ in Centre d'Etude du

Table 2

Whole-gene rare variant burden analysis per country

Country	Rare alleles/total alleles AD patients	Rare alleles/total alleles control individuals	Fisher exact (p value)
Belgium	14/870 (1.6%)	24/1744 (1.4%)	0.61
Spain	15/658 (2.3%)	21/968 (2.2%)	0.87
Italy	10/420 (2.4%)	11/1036 (1.1%)	0.09
Portugal	5/214 (2.3%)	6/254 (2.4%)	1.00
Sweden	6/350 (1.7%)	6/680 (0.9%)	0.24
Meta-analysis	50/2512 (2%)	68/4682 (1.5%)	OR = 1.32 (95% CI = 0.91–1.91) p value = 0.14 Heterogeneity – p value = 0.6

All nonsynonymous rare alleles were taken into account to perform the burden analysis. Fisher exact 2-tailed *p* values are shown for the individual populations. Mantel-Haenszel summary OR and Woolf test for heterogeneity are shown for the meta-analysis of the 5 cohorts.

Key: AD, Alzheimer's disease; CI, confidence interval; OR, odds ratio.

Polymorphisme Humain [Utah residents with ancestry from northern and western Europe] [CEU] population). Conditional logistic regression was performed to investigate if the observed association between AD and these common variants was mediated by the borderline effect of the low-frequency variant p.E274D (OR = 1.22 [95% CI = 1.01–1.47], nominal allelic *p* = 0.04) or the presence of rare alleles (OR = 1.23 [95% CI = 1.02–1.49], nominal allelic *p* = 0.034). None of these conditions could affect the association with AD.

3.4. Replication analyses in the EU EOD cohort

To increase power to interpret the findings of the Flanders-Belgian AD cohort, we extended our analysis to the EU EOD cohort, including 926 patients and 1476 control individuals originating from Spain, Portugal, Italy, Sweden, Germany, and Czech Republic. In total, 48 variations, both synonymous and non-synonymous, were identified in the exonic sequence of the *SQSTM1* gene. Of these, 44 variants were rare (MAF <1%) of which 23 caused a change at the protein level, 4 in AD patients only, 9 in controls only, and 10 in both patients and controls (Fig. 1, Supplementary Tables 2 and 3). Of the 4 variants that were only identified in AD patients and excluded from the tested control population, 2 variants were never described before in the context of PDB, ALS, or FTLD: p.P29S and p.L268V (Table 3). The patient carrying the p.P29S mutation also carried a second *SQSTM1* variant (p.A117V) and a pathogenic mutation in the *PSEN1* (p.L392V) gene, which most likely explain the early onset age of 40 years. Furthermore, the AD patient who carried the p.L268V mutation also carried another

mutation (p.P397L) that was also excluded from the control population but was earlier described in context of PDB.

Rare variant (MAF <0.01) burden analysis did not reveal an increased frequency of rare variants in *SQSTM1* in EOAD patients in any of the separate study populations nor when meta-analyzing all EU EOD cohorts of the consortium (OR = 1.39 [95% CI = 0.89–2.17], *p* = 0.14) (Table 2). Inclusion of the Flanders-Belgian cohort in the meta-analysis did not change the outcome (OR = 1.32 [95% CI = 0.91–1.91], *p* = 0.14) (Table 2). Furthermore, we found no evidence of predominant clustering of disease-causing alleles in specific protein domains in separate cohorts or in a meta-analysis with or without inclusion of the Flanders-Belgian cohort (data not shown). Meta-analysis of the low-frequency variant p.E274D (and p.S318=, in strong LD) in the different EU EOD cohorts did not reach statistical significance (OR_{p.E274D} = 0.9 [95% CI = 0.6–1.34], allelic *p* = 0.59). Inclusion of this variant in the whole-gene burden meta-analysis of the Flanders-Belgian and EU EOD cohorts (OR = 1.14 [95% CI = 0.89–1.46], allelic *p* = 0.28) did not change the observations. Remarkably, 17 of 29 synonymous variants were predicted to be “disease causing” by MutationTaster. However, inclusion of these variants in the rare variant meta-analysis did not show evidence of association with AD (OR = 1.15 [95% CI = 0.87–1.53], allelic *p* = 0.32). The common variants p.D292= and p.R312= showed association with AD (OR_{p.D292=} = 1.11 [95% CI = 1–1.22], nominal *p* = 0.04) (Fig. 2) but only when including the Flanders-Belgian cohort. Meta-analysis excluding the Flanders-Belgian cohort did not show evidence of association (OR_{p.D292=} = 1.07 [95% CI = 0.95–1.21], *p* = 0.27).

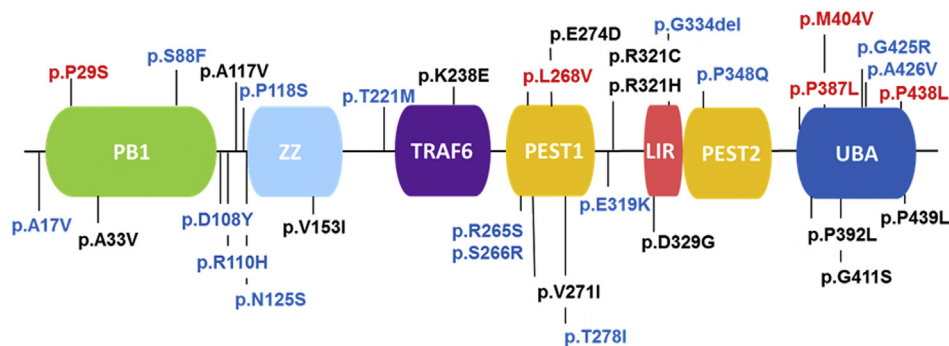


Fig. 1. Nonsynonymous sequestosome 1 (*SQSTM1*) mutations identified in Alzheimer's disease (AD) and control cohorts from Flanders-Belgian population and the European early-onset dementia consortium. Protein domains are indicated (transcript level on NM_003900.4 and protein level on the GenPept Accession Number NP_003891.1). Protein domains were assigned as described previously (van der Zee et al., 2014). PB1, PhoX and Bem1P; ZZ, zinc finger (zz type); TRAF6, tumor necrosis factor receptor-associated factor 6; PEST, regions rich in proline, glutamate, serine, and threonine; LIR, LC3-interacting region; and UBA, ubiquitin associated. Variants that were only identified in AD patients (*n* = 5) in our study are indicated in red. Variants that were only identified in control individuals (*n* = 15) in our study are indicated in blue. Variants identified in both AD patients and control individuals (*n* = 12) are indicated in black.

Table 3*SQSTM1* mutations present in patients and absent from the control cohorts that were screened for this study

Mutation	Functional domain	Origin	Gender	Clinical diagnosis	Family history	Age at onset (y)	Previously reported
p.P29S ^a	PB1	Italy	f	Definite AD	S	40	No
p.L268V	PEST1	Italy	f	Probable AD	S	58	No
p.P387L	UBA	Italy	f	Probable AD	S	58	FTLD/PDB
p.M404V	UBA	Italy	m	Probable AD	S	52	PDB
p.P438L	UBA	Belgium	f	Probable AD	F	67	SALS
			f	Probable AD	F	75	

Protein domains were assigned as described previously (van der Zee et al., 2014). More information on the AD patients carrying the mutations can be found in the columns “origin,” “gender,” clinical diagnosis,” “family history” (sporadic [S] or familial [F]), and “age at onset.” The column “previously reported” shows the variants that were previously described in context of ALS, FTLD, or PDB (Le Ber et al., 2013; Rea et al., 2013; Rubino et al., 2012). Rare variants p.L268V and p.P387L were carried by the same AD patient, originating from Italy.

Key: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; F, familial; f, female; FTLD, frontotemporal lobar degeneration; m, male; PB1, Phox and Bem1p; PDB, Paget disease of the bone; PEST1, proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST); S, sporadic; SALS, sporadic amyotrophic lateral sclerosis; *SQSTM1*, sequestosome 1; UBA, ubiquitin associated.

^a Carried a known pathogenic mutation for AD (presenilin-1 [*PSEN1*] p.L392V).

4. Discussion

In this study, we have investigated the presence of common and rare exonic variants in *SQSTM1* in a total of 1361 early-onset and/or familial AD patients and 2348 healthy individuals from 7 countries across Europe. We detected a total of 61 variants in the exonic region of *SQSTM1*, of which the majority ($n = 57$) were rare and identified in only one or few individuals, suggesting a high genetic variability of *SQSTM1*. We identified 5 variants that were not present in our tested control population of which one (p.P438L) was earlier described in the context of ALS (Rubino et al., 2012). Two variants, (p.P29S and p.L268V) that were only identified in our AD population, were excluded from publicly available databases (Exome Variant Server, dbSNP, and Ensemble). Overall, however, rare *SQSTM1* variants were identified at equal frequencies in AD patients and control individuals across populations (cumulative frequencies ranging from 0.9% to 2.8%), suggesting no major causal role for rare *SQSTM1* variants in the pathogenesis of early-onset AD. Of note, 2 of the variants we identified in patients only are known to be pathogenic in PDB (Rea et al., 2013). Other known pathogenic mutations for PDB were identified both in AD patients and control individuals, and the frequency of these mutations corresponded to the prevalence of PDB in the general population (1%–2%) (Ralston et al., 2008). Unfortunately, our patient cohorts were not

systematically screened for clinical or radiological signs of PDB, precluding further inferences.

Two AD patients harbored multiple rare variants in *SQSTM1*, and 2 patients carried both a *PSEN1* and a *SQSTM1* mutation. Double *SQSTM1* mutations were described earlier in the context of PDB (Collet et al., 2007) and ALS (Shimizu et al., 2013). This could imply that individual mutation burden of *SQSTM1* could modify disease susceptibility; however, additional systematic screening efforts are required to investigate this further. Of note, 2 control individuals also carried several *SQSTM1* variants.

Resequencing of the full coding region of *SQSTM1* revealed only 4 variants at individual frequencies >1%. Two common synonymous variants, which are in strong pairwise LD, showed marginal evidence of association with AD. These variations exert no obvious effect on protein, but in silico predictions (MutationTaster, Schwarz et al., 2014) suggest that they might introduce a splice site. Both SNPs are in pairwise LD with the GWAS top SNP rs72807343 ($D' = 1$), although a large difference in frequency of occurrence was found ($r^2 = 0.011$). However, the observed association appeared limited to the Flanders-Belgian population and would not have survived correction for multiple testing. Moreover, although the GWAS top SNP was not covered by the genotyping assays in the present study because of its localization outside the coding sequence of *SQSTM1*, it had previously been genotyped by custom Illumina SNP chip in the replication stage of an AD GWAS meta-analysis in part of our Flanders-Belgian late-onset AD cohort (887 AD patients and 674 control individuals; overlap with the patient cohort described here $n = 343$) (Lambert et al., 2013). In this subset of the Flanders-Belgian population, rs72807343 did not reveal statistical association with AD (OR = 0.83 [95% CI = 0.45–1.54], allelic $p = 0.56$).

One low-frequent missense variant, p.E274D (MAF 2%), showed a trend toward association in the Flanders-Belgian AD cohort. Interestingly, this variant showed tentative evidence of association in the International Genomics of Alzheimer's Project exome chip data analysis, which is performed on late-onset AD patients and control individuals (S. van der Lee and C.M. van Duijn, personal communication 2014). Nevertheless, when meta-analyzing the EU EOD cohort, this trend toward association disappeared. Of note, the Flanders-Belgian patient group had a higher average onset age than the EOD cohorts because of inclusion of familial AD patients with onset >65 years. Conceivably, this might explain why we cannot confirm the Flanders-Belgian trend toward association between *SQSTM1* variants and AD in the EU EOD cohort, which should have sufficient statistical power (>90%) to detect a risk allele with MAF 2% and OR 1.67 at alpha level of 0.05. In line with this, the GWAS association at *SQSTM1* was predominantly based on late-onset AD (Lambert et al., 2013).

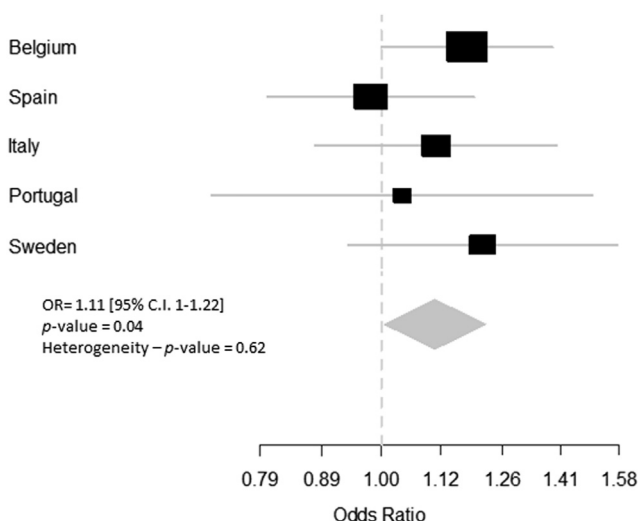


Fig. 2. Common variant meta-analysis of the Flanders-Belgian and European early-onset dementia cohorts: p.D292=.

In conclusion, in this European study on AD patients with early-onset and/or positive family history, thus likely to have an augmented genetic risk profile, we observed 61 variants in the exonic region of *SQSTM1* (comprising only 8 exons), both in patients and in cognitively healthy individuals, suggesting a high genetic variability of the gene. We cannot exclude a role of *SQSTM1* genetic variability in late-onset AD, but our data indicate that common and rare coding variations in *SQSTM1* do not play a major role in the etiology of early-onset AD.

Disclosure statement

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neurobiolaging.2015.02.014>.

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